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Frequency and immunophenotype of IL10-producing regulatory B cells in optic neuritis

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Summary

Mouse models of multiple sclerosis (MS) have shown the importance of interleukin-10 (IL-10) -producing regulatory B (Breg) cells in dampening disease activity and inhibiting disease initiation and progression. In MS and other autoimmune diseases decreased frequency and functionality of Breg cells correlate with disease activity and the percentage of IL-10-producing Breg cells decreases during relapse and normalizes in remission. Optic neuritis (ON) is a common first clinical manifestation of MS and IL-10-producing Breg cells may be crucial in the transition from ON to MS, we therefore investigate the frequency and function of Breg cells in ON as a clinical model of early demyelinating disease. B cells were purified from 27 patients with ON sampled close to symptom onset (median 23 days, range 7–41 days) and 13 healthy controls. The B cells were stimulated and cultured for 48 hr with CD40 ligand and CpG before measurement of intracellular IL-10 and the surface markers CD19, CD1d, CD5, CD24, CD38 and CD27 by flow cytometry. The frequency of B-cell subsets was analysed in peripheral blood and cerebral spinal fluid (CSF) of patients. Sixty-five per cent of the IL-10-producing Breg cells co-expressed CD24 and CD38, and only 14% were $CD24^{high}$ $CD27^{+}$, suggesting that the naive B cells are the primary source of IL-10 in the B-cell culture, followed by memory cells in both healthy controls and patients. The frequency of naive $CD19^{+}$ $CD24^{+}$ $CD38^{+}$ Breg cells was higher in patients with ON compared with controls. The ability of Breg cells to produce IL-10 was at normal levels in both ON patients with high risk and those with low risk of progression to MS. We found no correlation between Breg cell function and the presence of brain white matter lesions by magnetic resonance imaging or CSF oligoclonal bands indicative of ON patients carrying a higher risk of conversion to MS. The frequencies of IL-10-producing B cells did not correlate with the conversion to MS at 2-year follow up. Interleukin-10 was primarily produced by naive and memory B cells. The frequency of IL-10-secreting B cells did not correlate with risk factors of MS. Breg cell function at clinical onset of ON is not a determining factor for conversion to MS.

Keywords: clinically isolated syndrome; interleukin-10; multiple sclerosis; optic neuritis; regulatory B cells.

Abbreviations: Breg cells, regulatory B cells; CD40L, CD40 ligand; CIS, clinically isolated syndrome; CNS, central nervous system; CpG, Class B CpG oligonucleotide; CSF, cerebrospinal fluid; EAE, experimental autoimmune encephalomyelitis; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; FSC, forward scatter; GILTRL, glucocorticoid-induced tumour necrosis factor ligand; HC, healthy controls; IL-10, interleukin-10; MRI, magnetic resonance imaging; MS, multiple sclerosis; OCB, oligoclonal bands; ODN, oligodeoxynucleotides; ON, optic neuritis; PBS, phosphate-buffered saline; PE, phycoerythrin; PerCP, peridinin chlorophyll protein; PMA, phorbol-12-mystrate-13-acetate; PPMS, primary progressive MS; RA, rheumatoid arthritis; RRMS, relapsing–remitting MS; SLE, systemic lupus erythematosus; SPMS, secondary progressive MS; SSC, side scatter; WML, white matter lesions

Introduction

Multiple sclerosis (MS) is traditionally considered a $CD4^+$ T helper cell-mediated central nervous system (CNS) disease, where the inappropriate activation of T cells leads to demyelination and subsequent neuronal loss. In recent years, suppressive regulatory B (Breg) cells have been thought to play a role in the prevention of MS and other autoimmune diseases, such as inflammatory bowel disease,[1] systemic lupus erythematosus (SLE)[2] and rheumatoid arthritis (RA).[3] The Breg cells belong to several different lineages of B cells (mostly naive and immature memory B cells and plasmablasts)[4] and therefore Breg cells have been defined based upon their production of the anti-inflammatory cytokine, interleukin-10 (IL-10). It seems that IL-10 is responsible for most of the suppressive functions of the Breg cells. Breg cell-produced IL-10 inhibits the differentiation of pro-inflammatory T helper type 1 and type 17 cells and increases the differentiation of regulatory T cells, leading to dampening of the inflammatory response.[3] Moreover, IL-10 has been found to decrease antigen presentation by dendritic cells.[5,6] Apart from IL-10, some Breg cells also secrete glucocorticoid-induced tumour necrosis factor ligand and tumour necrosis factor-β, which regulates the proliferation of regulatory T cells and promotes recovery in animal models of autoimmune diseases.[7,8]

Several studies have investigated the frequency and function of Breg cells in MS patients. As IL-10-producing Breg cells belong to several different subsets of B cells rather than belonging to a specific lineage,[9] there is an ongoing search for B-cell subsets with the potential to produce IL-10. In humans, IL-10 is mainly produced by the memory B-cell ($CD19^+$ $CD24^{high}$ $CD27^+$) and naive B-cell subsets ($CD19^+$ $CD24^+$ $CD38^+$ $CD5^+$ $CD1d^+$ IgD^-).[10–12] Because of the lack of a good surface marker for immune phenotyping, different approaches have been used for identification of Breg cells. In peripheral blood, only small quantities of IL-10 are produced; hence, *in vitro* culturing and stimulation of B cells are necessary to study the IL-10 production by Breg cells. Some studies have used the CD5 and CD1d markers,[13,14] whereas others have used the CD24 and CD38 to estimate the number of nave Breg cells in humans,[2,15] and some of these studies have used IL-10 staining after *ex vivo* activation of B cells in addition to immunophenotyping. Breg cell activity can be interpreted as the capability to produce IL-10 when B cells are stimulated *in vitro*, most often by CD40 ligand (CD40) and Toll-like receptor 9 agonists alone or in combination with other stimuli. The amount of IL-10 produced and the subset of B cells producing IL-10 are largely dependent on the stimuli used.[10,16–18]

Compared with controls, most studies have found a reduced number of Breg cells in relapsing–remitting MS, secondary progressive MS and primary progressive MS.[17,19–21] However, in another study, no difference was observed[15] and a single study found an increased frequency of IL-10 Breg cells in a relatively small heterogeneous population of MS patients and healthy controls.[10] Michel *et al.* found normal IL-10 in response to stimulation in a cohort of patients with relapsing–remitting MS who were in remission, mainly consisting of newly diagnosed patients with low disease activity.[15] The different methods used and heterogeneity in the study cohorts probably explain some of these discrepancies. The Breg cell frequencies observed in MS patient samples were also found to be lower during relapse compared with remission[19] and were normalized during treatment with immunomodulatory drugs.[22,23] These studies indicate that the ability of Breg cells to produce IL-10 changes during disease progression, relapse and remission and may affect the severity and progression of MS.[19,21]

In contrast to studies in patients with MS, which so far have failed to provide conclusive evidence for the involvement of Breg cells in MS, several lines of evidence from models of autoimmune diseases suggest that IL-10-secreting Breg cells play a protective role in the early disease development of MS.[6] The depletion of B cells with anti-CD20 antibody (rituximab) or conditional knockout of IL-10 in B cells before induction of experimental autoimmune encephalomyelitis (EAE)[7] leads to more severe disease, whereas depletion of B cells during EAE progression has the opposite effect – dampening the symptoms.[24] This is in concordance with results from clinical trials where CD20-depleting rituximab and ocrelizumab reduce relapse rate and delay disease progression in MS.[25–27]

These experiments demonstrate that Breg cells reduce the severity of disease in an IL-10-dependent manner[28] and are essential for keeping pro-inflammatory T helper type 17 cells in check after relapse.[29] A few studies have investigated the effect of immunomodulatory treatment on Breg cell subsets. In SLE, treatment with rituximab leads to a higher ratio of immature to mature B cells[30] due to repopulation with immature B cells with IL-10 Breg cell potential.[20] Interferon-β, Copaxone and fingolimod increased the frequency of $CD24^+$ $CD38^+$ $CD5^+$ B-cell subsets as well as the production of IL-10 in patients with MS.[22,31]

In a model of myelin oligodendrocyte glycoprotein-induced EAE adoptive transfer of $CD1d^{hi}$ $CD5^+$, considered to be an IL-10-competent phenotype of B cell, into B-cell-depleted mice before immunization with myelin oligodendrocyte glycoprotein 35–55 inhibited leucocyte infiltration into the CNS and completely abolished EAE symptoms.[6,7] The transfer of pre-stimulated IL-10-

competent B cells before induction of EAE inhibited CNS infiltration of T cells and decreased demyelination.[24,32] The strong protective effect of IL-10 Breg cells in early EAE suggests that Breg cells might in particular be protective in the early phase of MS.[6]

Optic neuritis (ON) is a common first clinical sign of demyelination [clinically isolated syndrome (CIS)] in MS, and in approximately 50% of all cases, ON will progress to MS.[33] Given the importance of Breg cells in the initial stages of EAE, we investigated whether IL10 competent Breg cells are compromised during ON as a first clinical event. We compared the frequency and function of Breg cells between controls and patient, and correlated the Breg cell capacity to produce IL10 with the conversion to MS after two years. We found, that the Breg cell function in ON patients resembled that of healthy controls, and that the frequency of Breg cells did not correlated with the two year outcome after ON.

Materials and methods

Patient characteristics

All patients were recruited at the Clinic of Optic Neuritis and Multiple Sclerosis, Rigshospitalet, Glostrup, Denmark in the period April 2013 to April 2014. Patients aged between 18 and 59 years were included, and all were diagnosed with ON as a first demyelinating event. None of the patients received glucocorticoid treatment or immunomodulatory treatment before their lumbar puncture and venepuncture. The blood and cerebrospinal fluid (CSF) were sampled simultaneously and < 45 days (median 23 days, range 7–41 days) from symptom onset. All patients underwent a thorough diagnostic programme including visual tests, visual evoked potentials, magnetic resonance imaging (MRI) and analysis of CSF oligoclonal bands (OCB), IgG index and CSF leucocyte count. The MRI of brain and cervical column was performed on a Philips Achieva 3 Tesla system with Gadovist (Gd) enhancement studies. The presence and number of white matter lesions (WML) were assessed on the axial T2-weighted fluid attenuated inversion recovery (FLAIR) sequence. All patients underwent a neurological examination and other causes of ON were excluded through blood and CSF screening. As healthy controls (HC) we included 13 healthy blood donors from the Capital Region Blood Bank, Department of Clinical Immunology, Rigshospitalet, Denmark. Blood/buffy coats were provided for the study within 3 hr from blood donation.

Purification and in vitro stimulation of B cells

B cells were purified by negative selection from 40 ml whole blood or 10 ml buffy coat using RosetteSep™ Human B-cell Enrichment Cocktail (StemCell Technologies, Grenoble, France) according to the manufacturer's recommendations. The enriched B cells were collected and washed twice in phosphate-buffered saline (PBS; Apoteket, Rigshospitalet, Glostrup, Denmark) containing 2% fetal bovine serum (FBS; Biochrom Ag, Berlin, Germany). The purity of the cells was analysed by flow cytometry by staining for CD19-fluorescein isothiocyanate (FITC), CD20-phycoerythrin (PE) -Cy7, CD14-Peridinin chlorophyll protein (PerCP)-Cy5.5, CD3-V500 and PE-CD45 (all from BD Biosciences, San Jose, CA). The percentage of B cells at the time of culturing was 75·1 ± 13·5% (mean ± SD). There were no CD3-positive T cells in the cultures.

For analysis of the intracellular cytokine production by purified B cells, 100 000 cells/well were cultured in RPMI-1640 medium with ultra-glutamine and 25 mM HEPES (Lonza, Basel, Switzerland) supplemented with 10% FBS on a 96-well plate at 37° in a humidified 5% CO_2 incubator for 48 hr with or without stimulation. Cells were stimulated with 3 μg/ml CpG-B DNA (ODN 2006, Oligodeoxynucleotides; (Hycult Biotech, Uden, the Netherlands)), 1 μg/ml CD40L with CD40Enhancer (Enzo Life Sciences Inc., Farmingdale, NY), and 50 ng/ml phorbol-12-mystrate-13-acetate (PMA; Enzo Life Sciences Inc.) and 500 ng/ml ionomycin (Enzo Life Sciences Inc.) was added to the cell cultures for the last 4 hr of incubation. In addition, 0·3 μl GolgiStop™ (BD Biosciences) protein transport inhibitor was added to all cell cultures for the last 4 hr of incubation.

Flow cytometric analysis of IL-10-secreting B cells

Flow cytometric analysis of IL-10-expressing B cells was performed on 100 000 cells. The cells were washed twice in PBS with 2% FBS (FACS-PBS) and stained with 0·5 μl Live/Dead Fixable Viability Dye e506 (eBioscience Inc., San Diego, CA) for 30 min at 4°. Unspecific binding was blocked by adding 10% human serum. Cells were stained for extracellular surface markers; CD19-PerCp-Cy5.5 (HIB19; BioLegend, San Diego, CA), CD1d-allophycocyanin (51-1; BioLegend), CD5-FITC (L17F12; eBioscience), CD24-PE-Cy7 (ML5; BD Biosciences) and CD38-BV421 (HIT2; BD Biosciences) for 30 min in the dark at 4°. Cells were washed in FACS-PBS. After staining for the surface markers, the cells were fixed by adding 1 ml of Foxp3 Fixation/Permeabilization working solution (eBioscience) and pulse vortexed. The cells were left at 4° over night. Cells were washed in Permeabilization Buffer (eBioscience) and stained with IL-10-PE (JES3-9D7; eBioscience) for 45 min at 4° in the dark. After staining, cells were washed in Permeabilization Buffer, resuspended in 300 μl FACS-PBS and analysed on a FACSverse multicolour flow cytometer with FACSuite software (BD Bioscience). Fluorescence minus one controls were prepared

for each fluorescence label. A staining control of intracellular labelling of IL-10 (Isotype-PE) was included. Isotype staining and fluorescence minus one controls were used to define the gates. Cells were gated according to forward-scatter/side-scatter (FSC/SSC) criteria, followed by an FSC-A/FSC-H to ensure single cells. B cells were gated on the CD19 marker and IL-10-expressing B cells were further sub-gated into CD24 and CD38 as well as CD1d and CD5. The $CD24^+$ $CD38^+$ population was analysed for co-expression of CD1d and CD5.

Staining of surface markers in blood and CSF

B-lymphocyte and T-lymphocyte distribution and frequencies were examined in whole blood and CSF. In brief, 50 µl fresh whole blood from patients and 50 µl fresh buffy coat from HC were lysed with 1 ml FACS™ Lysing Solution (BD Biosciences), cells were washed with FACS-PBS + 2% FBS. Then, 100 µl 10% human serum in PBS was added to block unspecific binding. CSF from patients was collected on an ice bath. Ten millilitres CSF was centrifuged for 10 min at 400 ***g***, the cells were subsequently blocked for unspecific binding by adding 10% human serum directly to the CSF sample. Cells from blood and CSF were stained for 30 min at 4° in the dark with fluorescence-labelled antibodies for extracellular markers of B and T lymphocytes CD19-PerCp-Cy5.5 (HIB19; BioLegend), CD3-V500 (UCHT1), CD4-allophycocyanin (RPA-T4), CD8-FITC (SK1), CD20-PE-Cy7 (2H7) and CD45-PE (HI39), all from BD Biosciences. Blood and CSF were also stained with the same panel as the *in vitro* stimulated cultures, for details see above. Isotype-matched controls for each fluorochrome were included to confirm the specificity of the primary antibody. After staining, blood cells were washed twice and CSF cells were washed once in FACS-PBS + 20% FBS, resuspended in 300 µl FACS-PBS, and the lymphocytes were gated according to FSC/SSC criteria.

ELISA

Interleukin-10 secretion during *in vitro* B-cell stimulation was measured in the culture supernatants by human IL-10 enzyme-linked immunosorbent assay (ELISA; BioLegend, San Diego, CA); 120-µl aliquots of cell-free supernatant were frozen at −80° for subsequent analysis of IL-10. The IL-10 ELISA analysis was performed according to the manufacturer's recommendations. The ELISA plate was prepared by washing with washing buffer and blocking solution was added. The supernatants were brought to room temperature and diluted 1 : 3 or 1 : 5 in dilution buffer to meet the range of the assay, all samples and standards were measured in duplicate. The samples were incubated for 2 hr shaking. The plate was washed four times with washing buffer, before adding detection antibody for 1 hr. Following subsequent washes, the Avidin/horseradish peroxidase solution was added for 30 min, after which the plate was washed five times with washing buffer. The plate was developed by TMB substrate and the reaction was stoped with 2 N H_2SO_4. The absorbance was measured at 450 nm on a Tecan Infinity M200 (Tecan Life Sciences, Männedorf, Switzerland). The data were fitted by four-parameter curve fitting.

Statistics

The purity testing of the cell culture was analysed using Student's *t*-test. All other continuous variables are stated as median and range. For correlation analysis Spearman's rank correlation was used due to sample size. Although sample size was small, subpopulation data were found to approximate normal distribution, and so the Student's *t*-test for unpaired samples was used for comparisons between groups and the Student's *t*-test for paired samples was used for comparison between blood and CSF for patients. The level of significance was set to $P < 0{\cdot}05$. Statistical analysis was performed in SAS 9.2 (SAS institute Inc., Cary, NC, USA).

Ethics

All participants gave informed, written consent. The study was approved by the regional scientific ethics committee of Copenhagen, Denmark (protocol number H1-2011-019, H3-2010-055).

Results

Breg cells from patients with ON have normal capacity of IL-10 production

Dysregulated IL-10 production by Breg cells could influence the early progression of MS. We therefore investigated the IL-10-producing potential of B cells from HC and from patients with ON. A total of 30 patients and 13 HC participated in the study, one patient was later diagnosed with neuromyelitis optica and was excluded from the study. Although not all patients were included in all subparts of the study, the median age, gender ratio and frequency of patients with WML and OCB remained at the same level (Table 1). Since approximately half of patients with ON will progress to MS or be diagnosed with MS due to location and active lesions by MRI, we divided the ON patients into patients with no pathological findings besides the visual disturbances at diagnosis and those with signs of MS pathology, this latter group was named CIS/MS. A detailed demographic table of the patients and their paraclinical status at the time of ON diagnosis as well as the 2-year follow-up diagnosis can be

Table 1. Demographic and clinical characteristics of patients and healthy controls

	Patients *in vitro* studies	Healthy controls *in vitro* studies	Patients CSF cell profiling	Patient peripheral blood profiling
No. in study	16	13	18	27
Female/total ratio (%)	10/16 (62·5)	8/13 (61·5)	13/18 (72·2)	19/27 (70·4)
Age (interquartile range)	35 (27–38)	33 (28–43)	33 (27–40)	33 (26–40)
CSF leukocyte count ref $< 5 \times 10^3$/ml	9 (2–14)	N/A	7 (2–10)	N/A
IgG-index ref < 0·70	0·73 (0·43–0·83)	N/A	0·49 (0·44–0·77)	0·64 (0·41–0·77)
Oligoclonal band positive (%)	12/16 (75·0)	N/A	12/18 (66·7)	17/27 (63·0)
Pathological VEP	14/16	N/A	18/18	25/27
MRI T2 lesions [IQR]	3 (0–8)	N/A	1 (0–7)	1 (0–8)

This table presents age, gender, cerebrospinal fluid (CSF) leucocytes, IgG index, pathological visual evoked potential (VEP) and number of T2 lesion on MRI. N/A indicates that data were not available for healthy controls. All continuous variables are given as medians with interquartile range. Binary variables are given as percentage. For detailed demographic data consult the Supplementary material (Table S1).

found in the Supplementary material (Table S1). A total of 14 patients corresponding to 46·7% were diagnosed with MS at follow up, which is in line with previous reports on ON cohorts.[34]

Purified B cells were stimulated for 48 hr with CpG and CD40L plus addition of PMA and ionomycin the last 4 hr of stimulation and compared with unstimulated controls. As expected, unstimulated B cells from HC or from patients did not produce any measurable amount of IL-10 as investigated by flow cytometry (Fig. 1a–c). After stimulation, we found that 6·1% (1·2–23·0) [median (range)] of the B cells from HC, 2·0% (0·2–21·2) of the B cells from ON patients and 15·6 (4·0–32·5) in CIS/MS patients expressed IL-10. Although the CIS/MS B cells showed a tendency to higher percentage of IL-10-producing B cells this did not reach significance ($P = 0·06$) (Fig. 1c). We further investigated the secretion of IL-10 into the cell culture media after stimulation. The IL-10 concentration observed in the media correlated ($R = 0·51$; $P = 0·013$) with the percentage of IL-10 positive B cells observed by flow cytometry, suggesting that the IL-10 is indeed secreted from the cells (Fig. 1e). As observed by ELISA, no significant difference in the IL-10 concentration in the culture media was found between the patient groups and HC (Fig. 1d). We also stimulated cells with either CpG or CD40L alone or combined with the addition of PMA and ionomycin the last 4 hr of stimulation; these stimulations resulted in only marginal increases in IL-10 production (data not shown). Hence, our results demonstrate that B cells isolated from patients with ON as a first demyelinating episode have a normal ability to express and secrete IL-10 compared with B cells from HC.

IL-10$^+$ Breg cell numbers is not associated with markers of MS risk

The risk of MS development after ON is associated with the presence of WML on MRI, elevated CSF leucocyte count, elevated IgG index and the presence of CSF OCB.[34] We wanted to test if these paraclinical markers, known to be indicative of higher risk for converting from ON to MS, were associated with the percentage of IL-10-producing cells observed. We associated the percentage of peripheral blood Breg cells with number of T2 lesions on MRI, CSF leucocytes, IgG-index and presence of OCB (see Supplementary material, Table S1), but no significant correlation was found (data not shown). The CIS/MS patients with an increased risk of developing MS had a tendency towards higher frequency of IL-10 Breg cells and higher IL-10 secretion compared with ON patients with a low risk of progression, although this did not reach significance ($P = 0·06$) (Fig. 1c,d). In addition, the frequency of IL-10$^+$ Breg cells in the patients at high risk of progressing to MS and in those who were diagnosed with MS within a 2-year period following ON (85% of patients in the CIS/MS group) resembled the Breg cell frequency in HC (data not shown).

Immunophenotypes of IL-10 Breg cells are identical in ON patients and controls

We investigated if the IL-10 Breg cells from ON patients and HC differed in their co-expression of surface markers previously shown to be associated with regulatory capacities of the immature B-cell subsets (CD24, CD38, CD5 and CD1d). After CpG/CD40L/PMA/ionomycin stimulation of cells, the CD19$^+$ IL-10$^+$ population was sub-gated for CD24 and CD38 expression (Fig. 2a,b). Strikingly, we found that most of the Breg cells were CD24$^+$ CD38$^+$ in both HC 56·1% (21·9–96·0) and patients 78·8% (58·1–97·7) [median (range)] with a significantly higher frequency of CD24$^+$ CD38$^+$ in the ON patients (Fig. 2b). We also analysed the expression of the surface markers CD1d$^+$ and CD5$^+$ on B cells stimulated with CpG/CD40L/PMA/ionomycin and found that 25% of IL-10-positive B cells

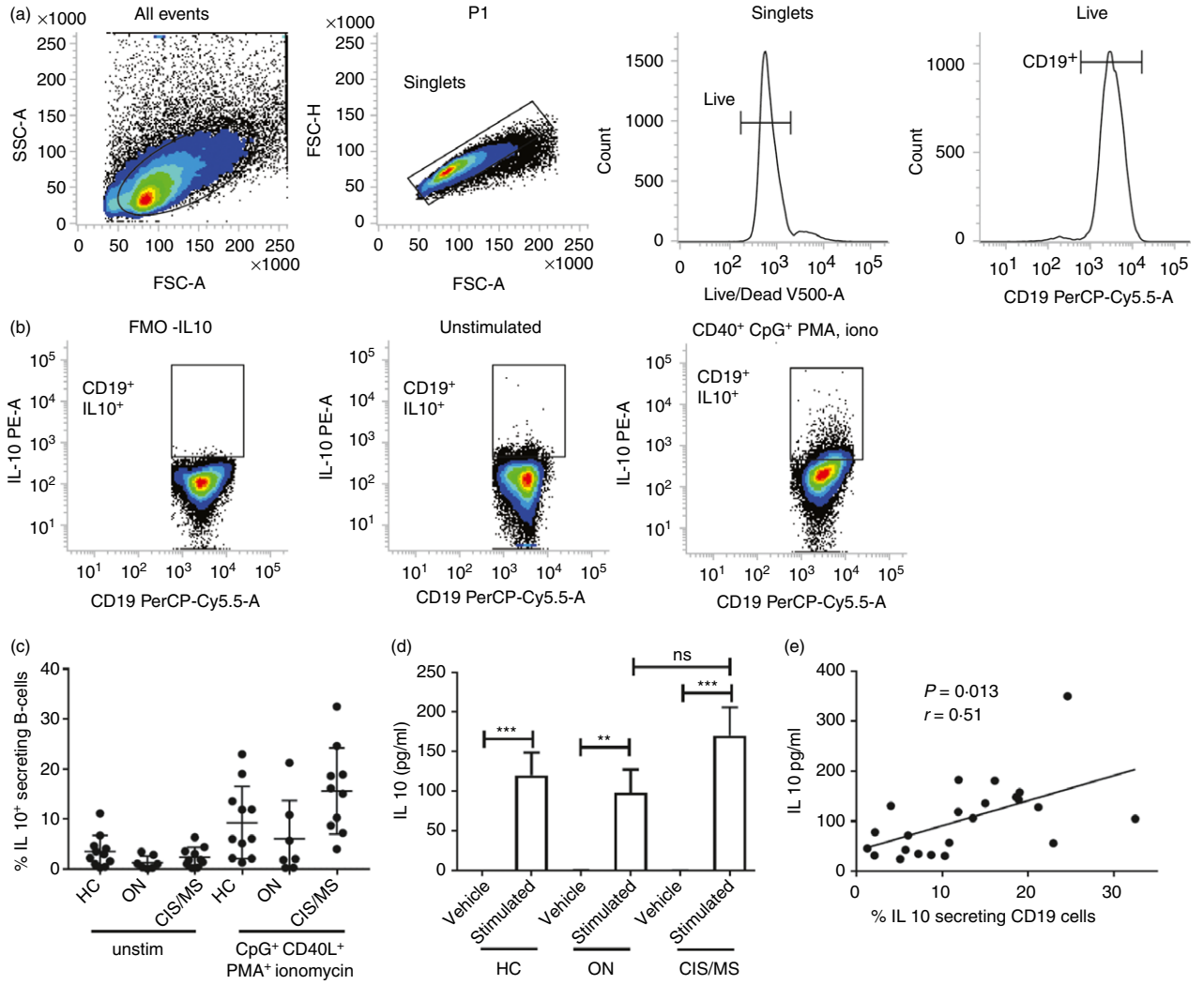

Figure 1. B cells from patients with optic neuritis have normal potential of producing interleukin-10 (IL-10). B cells purified from healthy controls (HC) or patients with clinically isolated syndrome (CIS) were cultured for 48 hr with CD40 ligND (CD40L) and CpG where PMA and ionomycin together with GolgiStop were added for the last 4 hr before analysis by flow cytometry. (a) Gating strategy, lymphocytes were gated from the FSC/SSC followed by a gating for singlets. Only live cells were included in the further analysis. B cells gated from $CD19^+$ cells. (b) IL-10-expressing B cells were gated based on fluorescence minus one (FMO) control. Representative plots of unstimulated cells and CD40L/CpG-stimulated cells are shown. Percentage of IL-10-secretion of unstimulated and CD40L, CpG, PMA and ionomycin stimulated B cells are shown for HC and ON patients without white matter lesions on MRI and CIS/MS patients diagnosed with MS at the time of ON or with MS-typical lesions on MRI (c). Release of IL-10 into the culture media after 48 hr (d). Correlation between the percentage of IL-10-positive B cells and the amount of IL-10 released from the B cells (e). $P < 0{\cdot}01$**, $P < 0{\cdot}001$***.

co-expressed CD1D and CD5, which was significantly higher than the 12·4% of $CD1d^+$ $CD5^+$ B cells in the whole B-cell population (Fig. 2c). We found no significant difference in expression of the surface $CD1d^+$ $CD5^+$ markers on Breg cells between the ON patients and the HC. The $CD1d^+$ $CD5^+$ Breg cells were found to be a subgroup of the $CD24^+$ and $CD38^+$ cells representing one-third of the $CD24^+$ $CD38^+$ Breg cells (Fig. 2d–f). Following stimulation, cells of the memory B cells ($CD19^+$ $CD24^{high}$ $CD27^+$) produced IL-10 and made up 14·3% of the Breg cells (Fig. 2g). The Breg cells were enriched among the $CD24^{high}$ $CD27^+$ B cells. Taken together, we find that the immunophenotype of Breg cells in patients is very similar to that of the HC, indicating that the IL-10-competent Breg cells in ON patients have neither been depleted nor expanded compared with the HC.

Recruitment of $CD24^{high}$ $CD27^+$ B cells into the CSF in ON

The distributions of B cells, CD4 T cells and CD8 T cells in the peripheral blood were comparable in patients with

ON and HC (see Supplementary material, Fig. S1). We further investigated whether B cells with a Breg phenotype and thus hold the potential to secrete IL-10 were recruited into the CSF of ON patients. We compared the surface marker phenotypes of B cells in peripheral blood of HC and ON patients, and the CSF of ON patients. Both in peripheral blood and CSF, the majority of $CD19^+$ cells expressed both CD24 and CD38, although this population was less abundant in CSF than in peripheral blood and comprised only of about 60% of the $CD19^+$ cells compared with 80% of B cells in peripheral blood (Fig. 3a). Further analysis of co-expression with the surface markers CD1d and CD5 showed that the fraction of $CD24^+$ $CD38^+$ cells expressing CD5 was lower in the CSF compared with peripheral blood (Fig. 3b–e), and $CD24^{high}$ $CD27^+$ memory B cells were enriched in the CSF compartment (Fig. 3f). In conclusion, although the CSF $CD24^+$ $CD38^+$ B cells were the largest population of IL-10-competent Breg cells in the *in vitro* stimulation assay, these phenotypes do not seem to be selectively recruited to the CNS. The IL-10 contribution from B cells in the CNS is more likely to reside from memory B cells recruited into the CSF.

Discussion

Regulatory IL-10-producing B cells play a significant role in suppressing autoimmune diseases such as MS, and studies in the MS model EAE point towards a particular importance of Breg cells during the early phases of disease, suppressing the disease initiation and development.[6,24] Patients with ON as a first demyelinating event are at risk as about 50% subsequently develop MS.[34,35] In particular, patients with WML on MRI and OCB in CSF are at high risk of developing MS over time.[34,35] In this study, 47% of all patients with ON and 87% of ON patients with a high risk of progressing to MS were diagnosed with clinical MS at 2 years following ON, which is in line with other studies on ON. Patients with ON as a first demyelinating event comprise a homogeneous patient group with a uniform symptomatology and so provide the opportunity to investigate the influence of Breg cell dysfunction on the early demyelinating pathogenesis in a clinical setting. To our knowledge, the function of Breg cells during ON has not been investigated previously. We found that Breg cell status at the time of first clinical symptom of ON did not predict the conversion to MS.

In the present study, we investigated the responsiveness of B cells isolated from ON patients close to disease onset and before receiving any immunomodulatory treatment or glucocorticoids. We stimulated cells *in vitro* with CD40L and CpG for 44 hr followed by a 4-hr re-stimulation with PMA and ionomycin. This stimulus led to IL-10 production in a fraction of the $CD19^+$ B cells to a similar degree in ON patients, CIS/MS patients and HC, indicating that at this early time-point of disease the function and frequency of IL-10 Breg cells were not impaired (Fig. 1c,d). Furthermore, a significant correlation between the release of IL-10 into the culture media and the frequency of $IL\text{-}10^+$ cells measured by flow cytometry indicated homogeneous production of IL-10 within the Breg cell subset (Fig. 1e). Because only a subset of ON patients progress into MS, we correlated B-cell IL-10 production with clinical data associated with a higher risk of progressing into MS after being diagnosed with ON.[34] Although we found a tendency for a higher frequency of Breg cells in patients with WML and OCB (CIS/MS group), it was not significant. A larger study is needed to conclude if B cells from these patients are more prone toward stimulation. The percentage of IL-10-producing Breg cells did not correlate to any of these risk factors, demonstrating that even in patients where ON is probably the first clinical symptom of MS, Breg cells apparently have a normal IL-10 response. The frequency of $IL\text{-}10^+$ Breg cells was not statistically different in HC and ON patients who were diagnosed with MS within a 2-year period following ON. In combination, these results indicate that a decrease in frequency or function of Breg cells was not crucial for the early progression of ON to MS.

Breg cells are challenging to study, because of the lack of a good surface marker. Hence, *in vitro* stimulation is commonly used to assess Breg cell functionality[2,3,6,10,17,19,20,36–40] and this is also the approach that we have used in this study. It is possible, that the *in vitro* stimulation protocol does not capture all the aspects of Breg cells influencing autoimmune pathophysiology and we can therefore not formally exclude that Breg cells function in ON is altered. Nevertheless, the *in vitro* stimulation protocol has been successfully used to demonstrate that Breg cell function is compromised in patients with MS,[13,16,17,20,21] as well as in other autoimmune diseases such as SLE[2] and RA,[3] suggesting that we would also have detected a reduced capacity for IL-10 production in ON patients if present. In particular, the frequency of IL-10-producing Breg cells has been found to be reduced during MS relapse compared with at remission[19,21] and after treatment with immunomodulatory drugs,[22,23] and several studies have found fewer IL-10-competent B cells in both relapsing–remitting MS, secondary progressive MS and primary progressive MS compared with controls.[17,19–21] Animal models of MS, suggest that the function of Breg cells and the number of B cells with Breg cell typical surface markers appear to play a significant role in delaying the disease initiation, whereas IL-10 from regulatory T cells becomes important in dampening disease later on.[6] Our data suggest that Breg cell function is not repressed during active disease during early MS. As we do not observe any correlation

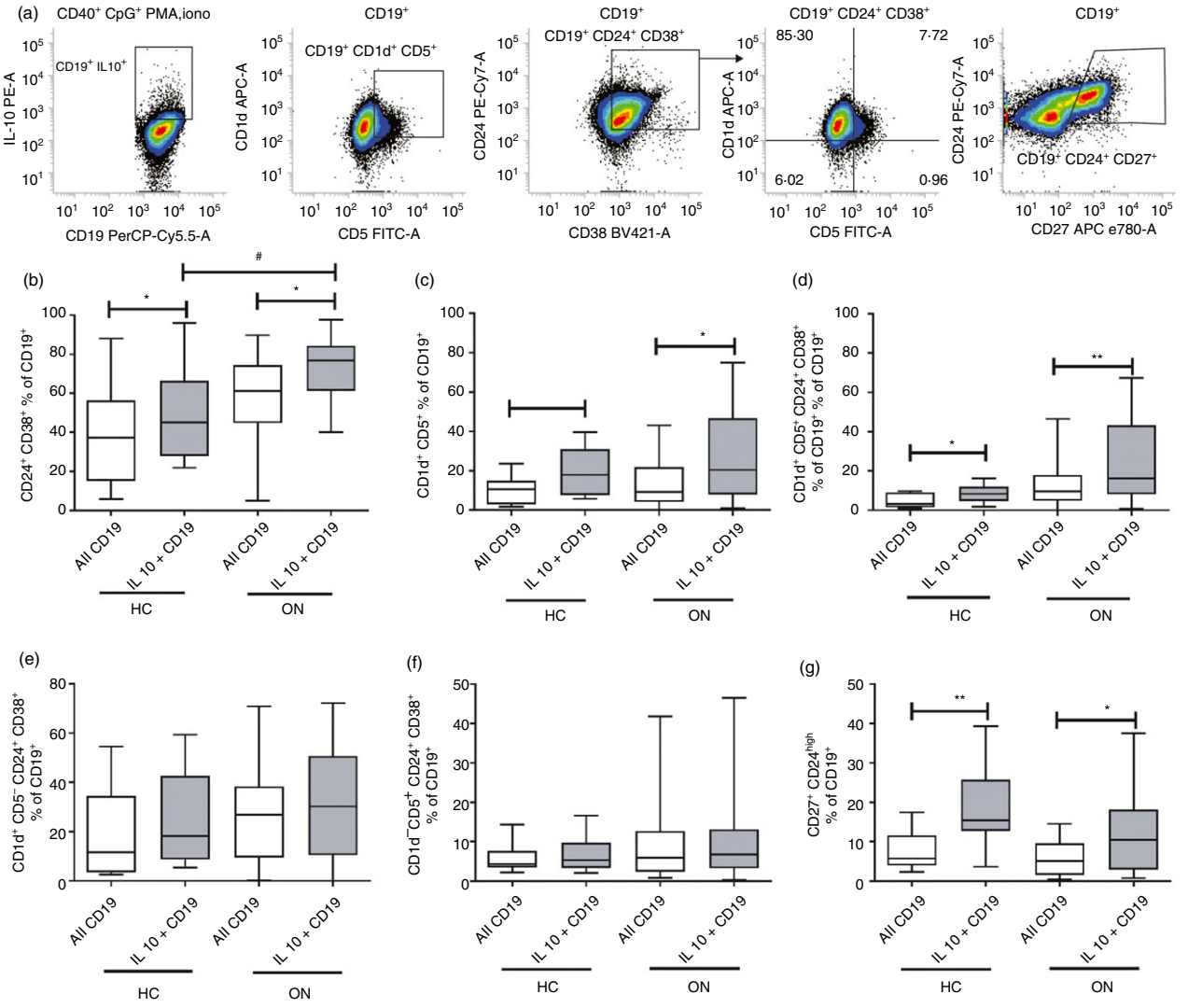

Figure 2. Gating strategy of IL-10$^+$ regulatory B (Breg) (a). CD19$^+$ IL10$^+$ cells were gated for CD24 CD38, the CD24$^+$ CD38$^+$ population was plotted for CD1d and CD5. The CD19$^+$ IL10$^+$ cells were also sub-gated for CD1d CD5. The expression of surface markers of CD19$^+$ IL10$^+$ was compared with the surface marker distribution of all CD19$^+$ B cells both in healthy controls (HC) and patients with optic neuritis (ON). Co-expression of CD24$^+$ CD38$^+$ (b), CD1d$^+$, CD5$^+$ (c), CD1d$^+$ CD5$^+$ CD24$^+$ CD38$^+$ (d), CD1d$^+$ CD5$^-$ CD24$^+$ CD38$^+$ (e) and CD1d$^-$ CD5$^+$ CD24$^+$ CD38$^+$ (f). Memory B cells were gated as CD19$^+$ CD24$^+$ CD27$^+$ (a,g). Overview of percentage distribution of phenotypes in all CD19$^+$ B cells and IL-10$^+$ B cells among all HC and patients. $P < 0{\cdot}05$*, $P < 0{\cdot}01$**.

between the Breg cell frequency in ON patients and the markers that predict progression to MS, it indicates that the mechanism responsible for the observed reduction in Breg cell capacity during later MS may be due to exhaustion of Breg cells over time. The recurrent immune attacks on myelin may, in an environment with fewer or dysfunctional Breg cells, not be able to dampen the immune reaction.

B cells produce IL-10 following stimulation by Toll-like receptors and activation of B-cell receptor by CD40L, the most prominent activation is Toll-like receptor 9 and B-cell receptor followed by a short stimulation with PMA and ionomycin.[10] In this study, we seek to link the surface marker phenotype in peripheral blood with the IL-10 production in ON patients and HC by investigating the expression of CD19, CD24, CD38, CD1d, CD5 and CD27, which have all previously been used to identify the Breg cell subsets.[2] The immature Breg cell subset is the primary IL-10-producing Breg cell subset in the peripheral blood following stimulation with CD40L.[3] We found that CD24$^+$ CD38$^+$ B cells are most prevalent among the IL-10-producing B-cell population (Fig. 2c), this is in line with percentages of CD24$^+$ CD38$^+$ IL-10$^+$ found by others using identical culture conditions.[41] We found that the CD24$^+$ CD38$^+$ B-cell population was predominant in the population of Bregcells, although the CD24^{high} CD27$^+$

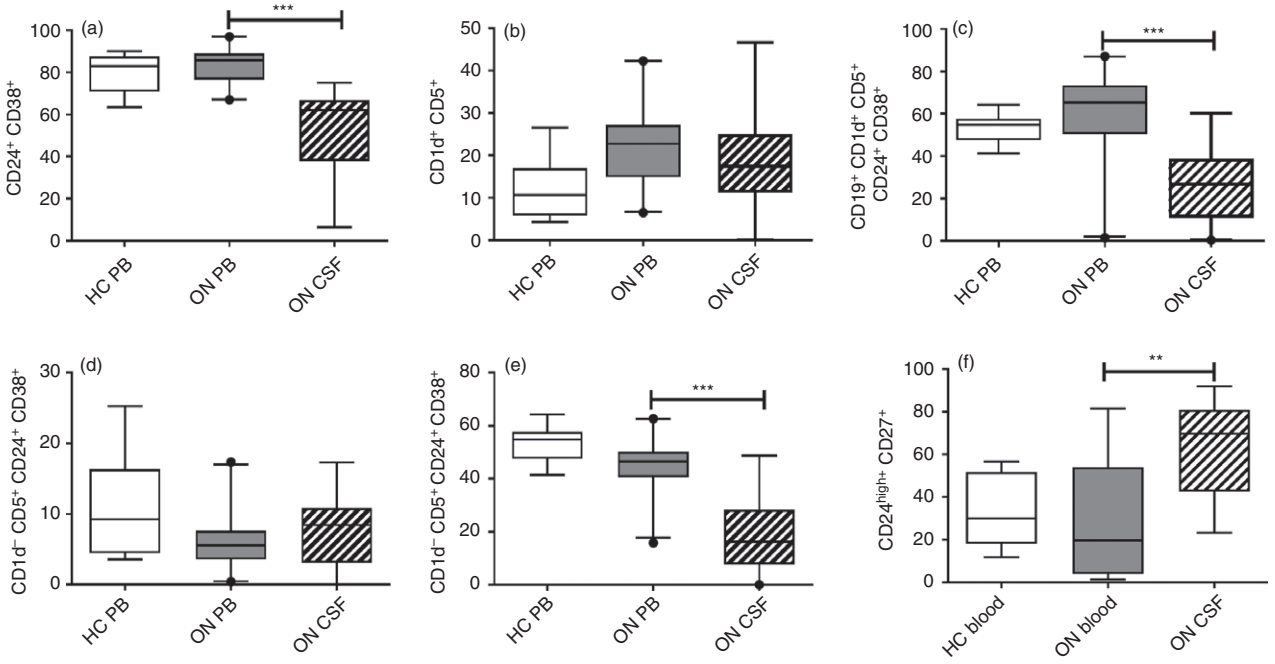

Figure 3. Surface marker expression of healthy controls (HC) (white) and patient B cells in peripheral blood (PB) (grey) and cerebrospinal fluid (CSF) (striped). Lower percentage of CSF B cells express $CD24^+$ $CD38^+$ compared with blood ($P < 0{\cdot}001$) (a). Equal fraction of $CD1d^+$ $CD5^+$ (b), and $CD1d^-$ $CD5^+$ $CD24^+$ $CD38^+$ (d) was found. Significant lower fractions $CD1d^+$ $CD5^-$ $CD24^+$ $CD38^+$ ($P < 0{\cdot}001$) (e) and $CD1d^+$ $CD5^+$ $CD24^+$ $CD38^+$ ($P = 0{\cdot}0014$) (c) were found in CSF. The frequencies of the populations in PB from HC and patients were comparable. Percentage variation for the phenotype is plotted in (f). Increased frequency of $CD19^+$ $CD27^+$ ($P < 0{\cdot}01$) in CSF compared with blood from ON patients. There was no significant difference in the distribution of phenotypes in PB from HC and ON patients (a–f). $P < 0{\cdot}01$**, $P < 0{\cdot}001$***.

memory B cells also produced IL-10 following stimulation. Knippenberg *et al.*[19] found in agreement with our data that the majority of IL-10 Breg cells belong to the naive B-cell population ($CD19^+$ $CD38^+$) and are the predominant source of IL-10. $CD1d^+$ $CD5^+$ naive B cells also have IL-10-producing capacities. In our study, all the $CD1d^+$ $CD5^+$ Breg cells co-expressed CD24 and CD38, hence $CD24^+$ $CD38^+$ were a better indicator of B cells with Breg-bearing potential (Fig. 2b–d). $CD5^+$ B cells have been found in more recently differentiated and highly proliferating B cells.[42] Clearly, the IL-10-competent Breg cells are expressing $CD24^+$ $CD38^+$ $CD1d^+$ and a subset of these also express CD5 (Fig. 2c–f). These data are in concordance with previous studies showing that IL-10 Breg cells belong to the $CD24^{high}$ $CD38^{high}$ population[2] and to the $CD1d^+$ $CD5^+$ population.[43,44] Stimulation of $CD5^+$ B cells by CD40L can lead to the production of IL-10,[45] but stimulation in our study only led to a smaller fraction of $CD5^+$ Breg cells (Fig. 2c–e). Interestingly in CIS patients a high percentage of $CD5^+$ B cells correlated with earlier conversion to MS,[13] pointing towards CD5 as a marker of more general activation of all B cells as well as towards a possible proliferation of this population in MS.[13] However, in RA, the percentage of $CD19^+$ $CD1d^+$ $CD5^+$ cells correlated negatively with disease activity.[23] The depletion of B cells positive for markers suggestive of B-cell IL-10 production during active disease, followed by rebound of these cell types during remission have been observed in MS and other autoimmune diseases.[2,46]Although the ON patients in our study had active disease, they had comparable levels to HC of all B-cell surface markers investigated. Memory B cells, $CD19^+$ $CD24^{high}$ $CD27^+$, hold the capacity to produce IL-10 in HC and in MS patients,[15,21] and this population is decreased in frequency during relapse compared with remission of MS.[14] In this study, we found equal distributions of the $CD19^+$ $CD24^{high}$ $CD27^+$ B cells in blood of patients and HC (Fig. 3f).

The local microenvironment and the recruitment of immune cells to the CNS of MS are crucial for the disease progression. In EAE the entry of Breg cells into the CNS largely dampens the disease severity.[47] We found in patients with ON that the frequency of $CD24^+$ $CD38^+$ B cells was lower in the CSF compared with the blood (Fig. 3a). Although the immature B-cell subtype only comprises a fraction of the total B cells in the CSF of MS patients, due to high numbers of plasma cells and memory cells,[48] immature B cells are still recruited into the CNS. In addition, we also confirmed the finding by Corcione *et al.*[48] of a higher percentage of $CD19^+$ $CD24^{high}$ $CD27^+$

memory cells in the CSF compared with peripheral blood (Fig. 3f).

In this study, we demonstrate that cells isolated from patients with ON have the same frequency of naive Breg cells and an equivalent capacity to produce IL-10, as cells isolated from HC. Furthermore, we find that the frequency of Breg cells does not correlate with the risk of progression to MS, as determined by the presence of MRI and CSF risk factors. Finally, we show that the Breg cells of HC and patients have similar immunophenotypes with $CD24^{+}$ $CD38^{+}$ being the predominant IL-10-producing cells followed by $CD24^{high}$ $CD27^{+}$ memory B cells. These results indicate that Breg cell function is not affected at the onset of MS but may become compromised in later stages of the disease by a mechanism that is not dependent on the Breg cell frequency at disease onset.

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Disclosures

The authors declare that they have no competing interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Patient demographic table, diagnosis and participation in experiments

Figure S1. Distribution of T and B cells in the peripheral blood of optic neuritis (ON) (white bars) patients, clinically isolated syndrome/multiple sclerosis (CIS/MS) (grey bars) patients and healthy controls (black bars).